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Fat intake modifies vascular responsiveness and receptor expression of vasoconstrictors: Implications for diet-induced obesity

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Abstract

Objective: Angiotensin II (Ang II), endothelin-1 (ET-1) and reactive oxygen species (ROS) have been implicated in the development of pathologic changes associated with obesity including hypertension and atherosclerosis. The aim of this study was to investigate the effects of dietary fat content on vasoreactivity and receptor expression at the level of gene and protein expression.
Methods: C57BL/6 mice were fed diets of normal (Control, 12.3% kcal from fat), high (HF, 41% kcal from fat) and very high (VHF, 58% kcal from fat) fat content for 15 weeks. Glucose tolerance tests were performed, and aortic rings were exposed to ET-1 (0.01–300 nM) and Ang II (100 nM) in the presence of L-nitro-arginine-methyl ester (L-NAME; 300 μM). Gene and protein expressions of angiotensin and endothelin receptors were examined by real-time PCR and immunoblotting, respectively. The effects of diet on responses to acetylcholine (ACh 0.1–300 μM), in the absence or presence of L-NAME, and to exogenous ROS/•OH were also investigated.
Results: Both high fat diets similarly impaired glucose tolerance ($P<0.05$). Increasing dietary fat augmented contractions to Ang II in a step-wise manner ($P<0.05$). Conversely, increasing dietary fat had no effect on contractions to ET-1. Exposure to ROS/•OH resulted in a rapid vasodilation that was markedly augmented in a step-wise manner with increasing dietary fat ($P<0.05$). Endothelium-dependent relaxation to ACh was unaffected whereas vasoconstriction to high concentrations of ACh was enhanced in VHF animals ($P<0.05$ vs. control). Gene expression of the AT_{1B} receptor was increased in the aorta of VHF mice, and aortic ET_A receptor protein expression was increased after both high fat diets.
Conclusions: These findings demonstrate that changes in dietary fat intake modulate vascular reactivity in response to Ang II and ROS, as well as expression of vascular angiotensin and endothelin receptors. Dietary fat intake may thereby directly affect cardiovascular risk.

Keywords: Hydroxyl; ROS; Artery; Comparative; Atherosclerosis; Vasoactive agents; Glucose tolerance; Vascular disease

1. Introduction

Dietary fat intake is a major determinant of the dramatic increase of obesity world-wide during the past two decades [1]. Obesity increases cardiovascular risk, significantly alters metabolic and cardiovascular function, and is associated with increased risk for diabetes, hypertension, and atherosclerosis [2,3]. Inflammation, vascular remodeling, and changes in vascular reactivity play a central role in the pathogenesis of these diseases [4–6].

Angiotensin II (Ang II) has been implicated in the development of hypertension and atherosclerosis [7,8]. The effects of Ang II are mediated via two receptor subtypes, AT_1 and AT_2; the AT_1 receptor consists of two isoforms, the AT_{1A} and the AT_{1B} receptor, which are functionally and pharmacologically indistinguishable [8]. Angiotensin II activates intracellular signalling pathways, primarily through the AT_1

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Table 1
Major macronutrient constituents of diets given in percent of kcal

Diet	Control	High fat	Very high fat
Protein	22.4	17	16.4
Carbohydrate	65.4	43	25.5
Fat	12.3	41	58

receptor, which promote atherosclerosis and hypertension through formation of reactive oxygen species (ROS) [9,10], inflammation [11,12], activation of cell growth [13], oxidative modification of lipoproteins [14], and by impairing endothelium-dependent vasodilation [8,15]. AT_1 receptor expression is increased in vascular smooth muscle cells of hypertensive patients [10]. In addition to its direct effects on the vasculature, Ang II also stimulates production of other vasoactive factors including endothelin-1 (ET-1) [16], a potent vasoconstrictor and mitogen [17] that binds to endothelin A (ET_A) and endothelin B (ET_B) receptors [18]. In pathological conditions such as atherosclerosis, ET_A receptors contribute to disease-progression [19,20]. Additionally, Ang II and ET-1 increase ROS generation [21,22], which include superoxide anion (O_2^-) and hydroxyl radical (•OH).

Changes in the vascular expression and/or activity of Ang II, ET-1 and ROS have been demonstrated in obesity, and preceed pathological changes associated with obesity [23–26], which in humans is often due to excessive dietary fat intake [1]. The aim of the current study, therefore, was to investigate the effect of diets of different fat content (41% and 58% fat) on vascular activity of Ang II and ET-1 and the expression of their receptors in fat-fed mice, a commonly used model of human obesity [27]. Moreover, the effects of ROS/•OH, and acetylcholine in precontracted aortic rings were investigated.

2. Methods

2.1. Animals and dietary treatments

Healthy male mice (C57BL/6, Charles River, Sulzfeld Germany) were housed in the institutional animal facilities on a 12:12-h light–dark cycle, and animals had free access to food and water. Housing facilities and experimental protocols were approved by the local authorities for animal research (Kommission für Tierversuche des Kantons Zürich, Switzerland) and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Mice were randomly assigned to one of the following diets (n=10–12 mice/group): control (12.3% of total kcal from fat, Kliba Nafag 3430, Kaiseraugst, Switzerland), high fat (HF, 41% of total kcal from fat, Research Diets D12079B, New Brunswick, NJ), and a diet containing very high amounts of fat (VHF, 58% of total kcal from fat, Research Diets D12331) for 15 weeks. The macronutrient compositions of the three diets are reported in Table 1. At the end of the treatment, mice were anesthetized (xylazine: 100 mg/kg body weight; ketamine: 23 mg/kg BW; and acepromazine: 3.0 mg/kg BW, i.p.), and exsanguinated via cardiac puncture. Blood was centrifuged at 5000 rpm at 4 °C for 15 min and plasma was stored at −80 °C.

2.2. Metabolic parameters and lipid measurements

In the week of the experiment mice were fasted overnight for 14 h, weighed, and venous blood was obtained from the tail vein (0 min) for baseline glucose measurements. Mice were subsequently injected (i.p.) with 2 mg/g BW D-glucose and blood was collected at 5, 10, 15, 30, 45, 60, 90, and 120 min. Blood glucose was determined with an AccuChek Advantage glucose meter (Roche Diagnostics, Switzerland). Plasma lipoproteins were determined enzymatically using a Cobas Integra 800 autoanalyzer (Roche Diagnostics, Rotkreuz, Switzerland), as previously described [28].

2.3. Vascular function studies

The thoracic aorta was isolated and placed in cold Krebs Ringer bicarbonate solution (in mmol/L: NaCl 118.6; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25.1; $EDTA_{Na2Ca}$ 0.026; glucose 10.1), dissected free of connective tissue under a microscope (Olympus SZX9, Volketswil, Switzerland) and cut into rings 3 mm in length. Special care was taken not to damage the endothelium during this procedure. Experiments were performed as previously described [29]. Vascular rings were mounted onto two tungsten wires (100 μm) and transferred to water-jacketed organ baths containing Krebs solution (95% O_2, 5% CO_2 at 37 °C, pH 7.4) and connected to force transducers (Hugo Sachs Elektronik, March-Hugstetten, Germany). Resting tension was gradually increased to the optimal level as previously determined in this laboratory (1.75 g) and aortic rings were repeatedly exposed to 100 mmol/L KCl until a stable response was achieved.

2.4. Vascular responses to angiotensin II and endothelin-1

Aortic rings were exposed to Ang II (100 nmol/L) [30] or ET-1 (0.01–300 nmol/L) [31] in the presence of the nitric oxide synthase inhibitor L-nitro-arginine methylester (L-NAME, 300 μmol/L) preincubated for 30 min.

2.5. Effect of ROS/•OH on precontracted aortic rings

Aortic rings were preincubated with L-NAME (300 μmol/L) for 30 min. The vascular response to exogenously generated reactive oxygen species (ROS), predominantly consisting of hydroxyl radical (•OH) was then investigated by simultaneous addition of vitamin C and Fe^{2+} (100 μmol/L each) [32] to rings of aorta preconstricted with phenylephrine to 50% of the KCl-induced contraction, as previously described [33]. The generation of hydroxyl radicals in the bath was confirmed by addition of terephthalic acid (TPA, 2.5 mmol/L).

Table 2
Primers used for amplification of a specific cDNA fragment encoding for ET_A receptor, ET_B receptor, AT_{1A} receptor, AT_{1B} receptor, AT_2 receptor and β-actin

Gene accession number	Forward primer reverse primer	Product size (bp)
ET_A receptor	5′-GAAGGACTGGTGGCTCTTTG-3′	149
BC_008277	5′-CTTCTCGACGCTGTTTGAGG-3′	
ET_B receptor	5′-CGGTATGCAGATTGCTTTGA-3′	189
NMU_32329	5′-CACCTGTGTGGATTGCTCTG-3′	
AT_{1A} receptor	5′-AGCCTGCGTCTTGTTCTGAG-3′	114
NM_177322	5′-ACTGGTCCTTTGGTCGTGAG-3′	
AT_{1B} receptor	5′-GCCTGCTAGTGACATGATC-3′	133
NM_175086	5′-GTACAGTCCAGAGTCCTTTC-3′	
AT_2 receptor	5′-CGCAGTGTGTTTAGAGTTCCC-3′	146
NM_007429	5′-AACCAATGGCTAGGCTGAC-3′	
β−Actin	5′-CGTGCGTGACATCAAAGAGA-3′	180
X_03672	5′-CCCAAGAAGGAAGGCTGGA-3′	

Hydroxylation of TPA yields a stable and highly specific isomer (monohydroxyterephthalate, TPA-OH) not directly produced from superoxide or hydroperoxides [34]. Fluorescence of TPA-OH was measured using a SpectraMax M2 (Molecular Devices, Basel, Switzerland) at 326 nM and 432 nM excitation and emission wavelength, respectively [35]. Addition of TPA to Krebs solution containing vitamin C and Fe^{2+} yielded 1804±178 versus 0.002±6.6 relative fluorescence units for Krebs solution alone ($P<0.005$).

2.6. Endothelium-dependent and -independent vasodilation

Rings were precontracted with phenylephrine (50% of KCl) and endothelium-dependent vasodilation was investigated using acetylcholine (ACh, 0.1 nmol/L–3 μmol/L) in the presence or absence of L-NAME (300 μmol/L). Endothelium-independent vasodilation was investigated using the nitric oxide donor sodium nitroprusside (SNP, 10 μmol/L).

2.7. Real-time PCR

Aortic tissue was snap-frozen in liquid nitrogen and stored at −80 °C. Tissue was pulverized and total RNA was extracted using the silica-based RNeasey Mini™ Kit (Qiagen, Hilden Germany). 150 ng RNA was reversed transcribed with Quantitect Reverse Transcription Kit™ (Qiagen, Hilden Germany), including a genomic DNA digestion step. Expression levels of the murine genes encoding for ET-1 receptors (ET_A and ET_B receptors) and AT_{1A}, AT_{1B} and AT_2 receptors were determined by real-time PCR as described [36]. Real-time PCR experiments were run on the iQ™ iCycler (Bio-Rad, Reinach, Switzerland) using specific cDNA primers (Microsynth, Balgach, Switzerland, Table 2). Murine β-actin was used as a house-keeping control.

2.8. Western blot analyses

For protein expression analysis, three pieces of tissue from each group were pooled and homogenized in RIPA lysis buffer. Equal amount of protein lysates were separated on an 8–16% SDS-PAGE gel and immunoblotted with anti-angiotensin receptor type I antibody and anti-ET-1 receptor antibody. Equal amount of protein loading was controlled by probing with an anti-p42/p44 antibody [37].

2.9. Materials and antibodies

ET-1 and L-NAME were supplied by Alexis Corp (Lausanne, Switzerland). All other chemicals were supplied by Sigma Chemicals Co. (Buchs, Switzerland). Antibodies against angiotensin II type I receptor, against p42/p44 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and anti-ET-1 receptor antibody from BD Transduction Laboratories (Franklin Lakes, NJ, USA).

2.10. Statistical analyses

Data are given as mean±SEM and n denotes the number of animals used. Contraction is expressed as a percentage of contraction to100 mmol/L KCl, and dilations are given as a percentage of the maximal contraction. EC_{50} values (as negative logarithm pD_2) were calculated with non-linear regression analysis and the area under the curve (AUC) was calculated for each individual curve using SigmaPlot (SPSS Inc. Chicago, IL). Data were analyzed using ANOVA for repeated measurements with Bonferroni correction, the unpaired Student's t-test or the Mann–Whitney U test, when appropriate. A P value <0.05 was considered significant.

3. Results

3.1. Weight gain and metabolic studies

After 15 weeks mice fed a control diet had gained 11±1 g of body weight, while the mice fed high fat and very high fat diets gained 17±1 g and 21±1 g, respectively ($P<0.004$ C vs. HF, $P<0.001$ C vs. VHF, $P<0.04$ HF vs. VHF). While the feeding of high fat diets for 15 weeks did not alter fasting

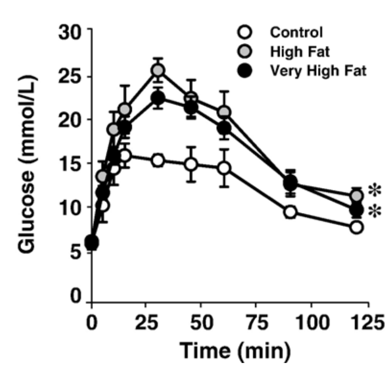

Fig. 1. Plasma glucose levels at base line (0 min) and at indicated time points after i.p. injection of 2 mg/g BW D-glucose (glucose tolerance test). Mice were fed diets containing control (C, 12.3%), high fat (HF, 41%) and very high fat (VHF, 58%) kilocalories from fat for 15 weeks. Data are means± standard error ($n=5$, C; $n=6$, HF; $n=12$, VHF). *$P<0.04$ vs. control diet.

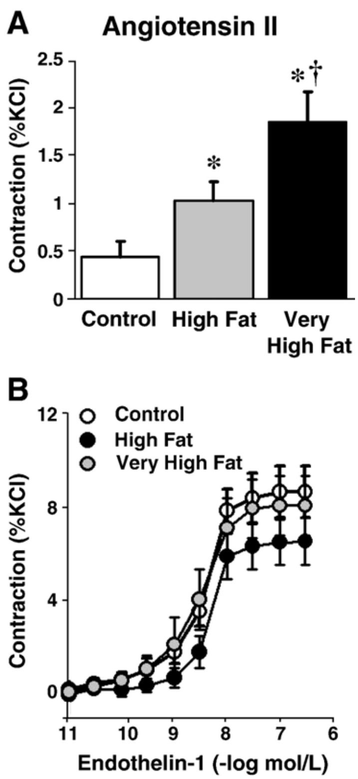

Fig. 2. Effects of dietary fat content on contraction to Ang II (100 nmol/L) in aorta (A). Dietary fat content increased vasoconstrictor responses to Ang II in aorta. $n=8$–15/group. *$P<0.04$ vs. control. †$P<0.04$ vs. high fat. Effects of dietary fat content on contractions to ET-1 in aorta (B). Increasing dietary fat and animal weight had no effect on ET-1-induced contractions in the aorta. $n=6$–12/group.

glucose (in mmol/L, C = 5.5 ± 0.2, HF = 5.2 ± 0.4, VHF = 5.9 ± 0.3), glucose tolerance was significantly impaired in both the 41% (HF) and 58% fat (VHF) diet fed mice ($P<0.04$ versus control, Fig. 1). Plasma cholesterol levels similarly increased after both high fat diets (in mmol/L, C = 2.1 ± 0.1, HF = 3.1 ± 0.2, VHF = 3.2 ± 0.2, $P<0.001$ HF and VHF vs. C).

3.2. Contractility to angiotensin II and endothelin-1

In the aorta, increasing dietary fat content enhanced contractions to Ang II in a concentration-dependent manner ($P<0.04$ HF vs. C; $P<0.002$ VHF vs. C; $P<0.04$ VHF vs. HF, Fig. 2A). Endothelin-1 caused concentration-dependent contractions that were unaffected by 15 weeks of high fat diets (Fig. 2B). The AUC values of contractions and sensitivity to ET-1 were also unaffected (Table 3).

Table 3
pD_2 values and area under the curve values (AUC, a measure of overall contractility) were calculated for each dose–response curve to ET-1 and data were averaged

Diet	Control	High fat	Very high fat
Aorta			
pD_2	8.4 ± 0.04	8.5 ± 0.1	8.5 ± 0.1
AUC	17 ± 3	13 ± 2	18 ± 3

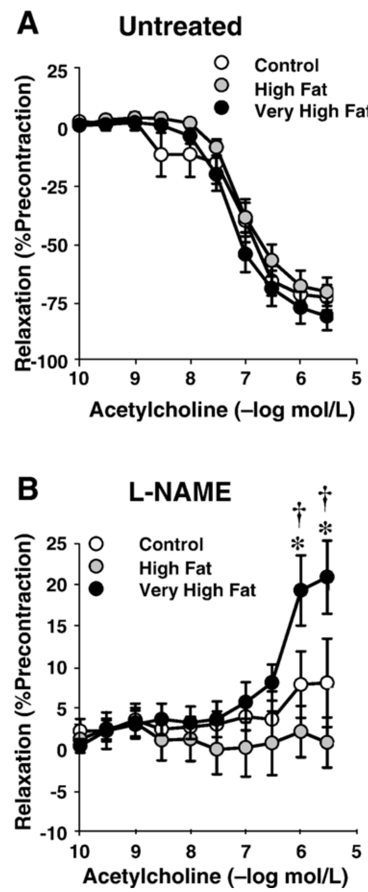

Fig. 3. Endothelium-dependent vasodilation to acetylcholine (ACh) in the aorta, in the absence (A) or presence (B) of L-NAME (300 μmol/L). While increasing dietary fat had no effect on vasodilation to ACh, vasoconstriction at high concentrations of ACh (≥ 1 μmol/L) in the presence of L-NAME was markedly increased in very high fat diet fed animals. $n=8$–12/group. *$P<0.05$ vs. control, †$P<0.05$ vs. high fat.

3.3. Endothelium-dependent and -independent vasodilation

Acetylcholine caused concentration-dependent relaxations, which was unchanged with increasing dietary fat content (Fig. 3A), and no difference in endothelium-independent

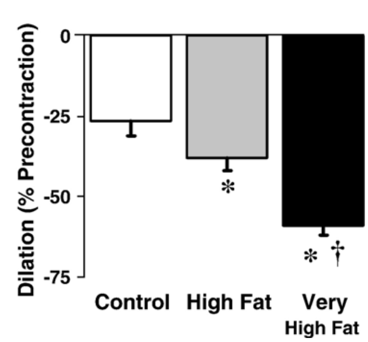

Fig. 4. Dilator responses to reactive oxygen species (ROS/•OH) in vascular rings precontracted with phenylephrine (to 50% of KCl). Increasing dietary fat content enhanced the vasodilation. $n=8$–12/group. *$P<0.05$ vs. control. †$P<0.05$ vs. high fat.

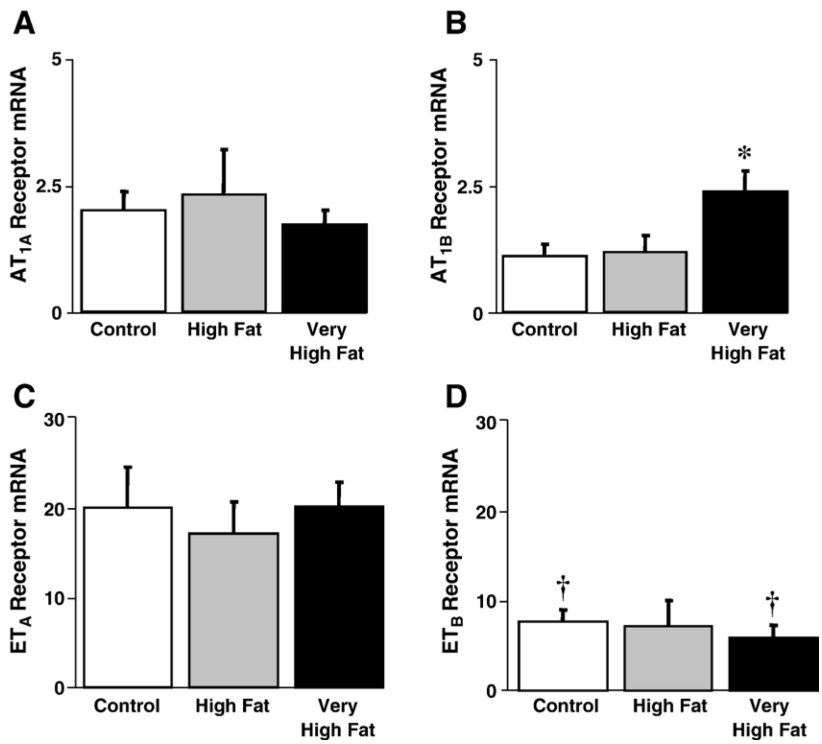

Fig. 5. Effects of dietary fat content on relative Ang II receptor mRNA expression levels in aorta. Increasing dietary fat content had no effect on AT_{1A} receptor expression, but increased the expression of AT_{1B} receptor expression in the very high fat diet group. $n=4$–6/group. $^*P<0.05$ vs. control. Effects of dietary fat content on relative ET-1 receptor mRNA expression levels in aorta (B). Increasing dietary fat content had no effect on ET_A or ET_B receptor expression. The expression of ET_A receptors was 3-fold greater than that of ET_B receptors ($P<0.05$). $n=4$–6/group. †$P<0.03$ ET_A vs. ET_B within a treatment group.

vasodilation to SNP between groups was observed (data not shown). Inhibition of nitric oxide synthase with L-NAME completely abolished the vasodilator response to ACh (Fig. 3B). Interestingly, at high concentrations (≥1 μM, Fig. 3B) ACh caused contractions in the VHF group ($P<0.004$ versus control) that were not seen in either the control or HF group.

3.4. Vascular responses to reactive oxygen species

Exogenously added ROS/•OH caused vasodilation in precontracted aortic rings ($P<0.0001$ vs. untreated). Increasing dietary fat augmented the vasodilator responses to ROS/•OH independently of nitric oxide synthesis, which was inhibited by L-NAME ($P<0.05$ C vs. HF and VHF, Fig. 4).

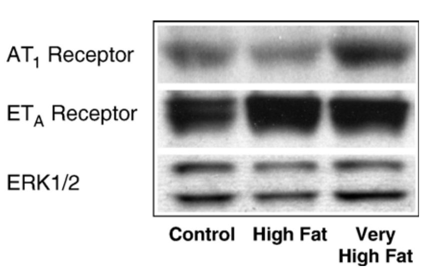

Fig. 6. Effects of dietary fat content on AT_1 and ET_A receptor protein expression in aorta. Tissue extracts were analyzed by immunoblotting with antibodies against AT_1 receptor, ET_A receptor and ERK1/2 as a loading control. Representative immunoblots are shown.

3.5. Gene expression of angiotensin and endothelin receptors

In mice fed a VHF diet vascular AT_{1B} receptor gene expression was increased compared to control diet fed mice ($P<0.03$ VHF vs. C, Fig. 5B). AT_{1A} receptor gene expression, however, was similar between groups (Fig. 5A). The AT_2 receptor gene could not be reliably quantified as it was expressed at very low levels close to the detection limit (amplification began around 34 PCR cycles, data not shown).

Endothelin A (ET_A) and endothelin B (ET_B) receptors were expressed in all samples investigated, and gene expression levels were similar between groups (Fig. 5C and D). ET_A receptor gene expression was approximately 3-fold higher than that of the ET_B receptor in each group ($P<0.03$ C and VHF, $P=0.067$ HF).

3.6. Protein expression of angiotensin and endothelin receptors

Immunoblot analysis of AT_1 receptor indicated no differences in expression between dietary groups (Fig. 6). However, protein expression analysis of ET_A receptor indicated an increase in receptor expression in the HF- and VHF diet fed mice as compared to controls (Fig. 6).

4. Discussion

The current study demonstrates, for the first time, differential effects of fat intake on aortic vascular reactivity to and receptor expression of vasoactive factors despite similar changes in glucose tolerance and plasma cholesterol. Increasing fat intake caused a step-wise increase in the vasoconstriction to Ang II and acetylcholine, while the vasodilator response to ROS was enhanced. High dietary fat intake was associated with increased vascular AT_{1B} and ET_A receptor expression.

Angiotensin II plays an important role as a trophic factor in the development of hypertension [38], and enhanced vasoconstrictor effects to Ang II and increased Ang II plasma levels have been reported in obese patients and animal models [24,39,40]. In the present study we observed that fat intake dose-dependently augments aortic contractile responses to Ang II, which in young mice is largely mediated by cyclooxygenase-1 [30], and similar changes were seen in the renal artery (Mundy and Barton, unpublished observation, 2006). Additionally, aortic AT_{1B} receptor gene expression increased in VHF mice as compared to controls, while AT_{1A} receptor gene expression remained unchanged. In mice, the AT_{1B} receptor mediates Ang II-induced vasoconstriction [41], and activation of the AT_1 receptor has been implicated in the development of atherosclerosis and hypertension [8]. The AT_1 receptor is also upregulated in leptin-deficient rats that spontaneously develop obesity [42]. The data of the present study suggest that enhanced sensitivity of the vasculature to Ang II and the increased receptor expression upon increasing dietary fat content are likely to facilitate the development of hypertension and vascular disease due to obesity.

Angiotensin II induces vascular ET-1 expression *in vivo* [16], and circulating levels of ET-1 are increased in obese patients [23,24] and in the kidney of obese mice [24]; we have now investigated the effects of different amounts of dietary fat intake for 15 weeks on the vascular responses to ET-1. Vascular contractions to ET-1 were unchanged after 15 weeks with either of the high fat diets and no effect on the expression of both ET_A and ET_B receptor mRNA was observed. In contrast, at protein level, ET_A receptor expression was increased in HF and VHF groups as compared to the control group. This increase in protein expression is likely to be mediated by post-translational modifications and/or changes in protein stability. The results of other studies have shown variable results depending on a number of factors, especially the duration of dietary intervention and vascular bed studied. For example, while increased contractions to ET-1 were observed in the aorta of mice fed a high fat diet for 30 weeks, contractions in the carotid artery were unaffected [29]. In the present study maximal contraction to ET-1 was also unaffected by 15 weeks of either the HF or VHF diet in both the renal and femoral arteries (Mundy and Barton, unpublished data, 2006). The shorter duration of dietary treatments used in the current study (15 vs. 30 weeks) and the vascular bed examined may explain the observed differences [29]. When assessing contractile responses, parameters such as receptor density, receptor affinity, signaling cascades mediating contraction (including calcium flux) could not be studied in our experimental set-up. However, endothelin is a potent trophic factor stimulating cell growth via the ET_A receptor [43]. Thus, upregulation of the ET_A receptor could promote accelerated myocardial and vascular hypertrophy, which are also known to occur in animals and patients with obesity [44].

Angiotensin II [9] and ET-1 [22] are both known to induce vascular generation of ROS, which include superoxide anion (O_2^-) and hydroxyl radical (•OH). In the present study we investigated the effects of ROS/•OH on the vasculature and changes of the responses by increasing fat intake. Although •OH is commonly perceived to be an "injurious" ROS [45,46], generated by the interaction of superoxide, hydrogen peroxide and iron (Fenton and Haber-Weiss Reactions), we have recently found that constitutively generated •OH also has vasodilatory effects, which are enhanced in the aorta of genetically obese mice [33]. In the current study, the dilatory effect of ROS/•OH increased depending on dietary fat, suggesting that high fat intake and/or obesity, enhance vasodilating properties of ROS/•OH. Remarkably, the vasodilator response to ROS/•OH was unaffected by the inhibition of NO synthesis in all study groups. This may represent a novel vasodilator back-up mechanism in states associated with high fat intake and/or obesity, as well as low NO bioactivity. In most forms of vascular diseases such as atherosclerosis, diabetes, aging and particularly in obese patients, bioactivity of NO is reduced, which is mimicked in our experimental set-up by the presence of L-NAME in the aortic rings [6,19].

No differences in the endothelium-dependent vasodilation to acetylcholine were observed between groups after 15 weeks of feeding. Previous studies have demonstrated impaired endothelium-dependent vasodilation to ACh in aorta of mice after 30 weeks of high fat diet treatment [29], and in aorta of rats after 2 years [47] or 8 weeks [48] of high fat feeding. Thus, differences are possibly due to variations in duration of dietary treatment. In the absence of nitric oxide after L-NAME treatment *in vitro*, however, a marked increase in the vasoconstriction to ACh was noted in the VHF group as compared with the control group. The vasoconstrictor response to ACh in mice is known to be caused by cyclooxygenase-dependent prostanoids [29,49], which increase with obesity [29]. Given our previous observation that endothelium-dependent relaxation is impaired after 30 weeks of high fat diet, our data suggest the possibility that changes in prostanoid activity in the early stages of obesity development precede overt impairment of endothelium-dependent vasodilation.

In conclusion, changes in fat intake specifically alter the reactivity to vasoconstrictor substances and ROS/•OH, accompanied by changes in angiotensin and endothelin receptor expression. As glucose tolerance and cholesterol levels were affected to a similar degree in mice fed either of the two high fat diets, the increase in the responses to Ang II and ROS/•OH are likely to be directly related to fat intake. The results suggest that, already at early stages of obesity development, the vasculature is sensitive to functional and expressional changes in response to modifications in dietary fat content. In view of the growth-promoting effect of Ang II and ET-1, and if applicable to human obesity, these results suggest important new roles for fat intake and obesity for vascular dysfunction and early development of cardiovascular disease [50,51].

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